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Possible Environmental Origin of Resistance of *Aspergillus fumigatus* to Medical Triazoles[▽]

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We reported the emergence of resistance to medical triazoles of *Aspergillus fumigatus* isolates from patients with invasive aspergillosis. A dominant resistance mechanism was found, and we hypothesized that azole resistance might develop through azole exposure in the environment rather than in azole-treated patients. We investigated if *A. fumigatus* isolates resistant to medical triazoles are present in our environment by sampling the hospital indoor environment and soil from the outdoor environment. Antifungal susceptibility, resistance mechanisms, and genetic relatedness were compared with those of azole-resistant clinical isolates collected in a previous study. Itraconazole-resistant *A. fumigatus* (five isolates) was cultured from the indoor hospital environment as well as from soil obtained from flower beds in proximity to the hospital (six isolates) but never from natural soil. Additional samples of commercial compost, leaves, and seeds obtained from a garden center and a plant nursery were also positive (four isolates). Cross-resistance was observed for voriconazole, posaconazole, and the azole fungicides metconazole and tebuconazole. Molecular analysis showed the presence of the dominant resistance mechanism, which was identical to that found in clinical isolates, in 13 of 15 environmental isolates, and it showed that environmental and clinical isolates were genetically clustered apart from nonresistant isolates. Patients with azole-resistant aspergillosis might have been colonized with azole-resistant isolates from the environment.

Invasive aspergillosis is a fungal disease caused by *Aspergillus* species that primarily affects immunocompromised patients, such as those treated for hematological malignancy. Patients may become infected by inhalation of ambient air that contains fungal spores. The *Aspergillus* conidia can penetrate into the alveoli and if not effectively removed, may germinate, proliferate, and cause invasive aspergillosis. Mortality and morbidity due to invasive aspergillosis remain a significant problem.

Triazoles, such as itraconazole (ITZ), voriconazole, and posaconazole, are used increasingly in the management of patients with this disease. Although the risk of resistance due to the increased use of triazoles is considered low (11), we recently observed ITZ resistance rapidly emerging in clinical *Aspergillus fumigatus* isolates (19, 22, 24, 25). Azole resistance was observed in up to 6% of patients in our hospital and in up to 14.5% of isolates sent to our laboratory from other hospitals in The Netherlands, which were obtained from patients with aspergillus disease (19). Furthermore, azole resistance has been reported in other European countries (3, 13, 19). The ITZ-resistant isolates also showed significantly reduced susceptibility to the other mold-active medical triazoles voriconazole and posaconazole (19). A substitution of leucine for histidine at codon 98 (L98H), combined with a 34-bp tandem repeat (designated TR) in the promoter region of the *cyp51A* gene (TR/L98H), which is the target for antifungal azoles, was found in 94% of isolates (14, 19, 24).

Azole resistance can develop through the exposure of the fungus to azole compounds, which may occur in azole-treated patients or through the use of azole compounds in the environment. The dominance of a single resistance mechanism is difficult to explain by resistance development in individual azole-treated patients, as one would expect multiple resistance mechanisms to develop. Also, spread by person-to-person transmission of any *Aspergillus* isolate is highly unlikely. As inhalation of airborne aspergillus spores is the common route of infection for aspergillus diseases, we hypothesized that the dominance of a single resistance mechanism in clinical ITZ-resistant isolates was more consistent with acquisition from a common environmental source (19). If azole-resistant *A. fumigatus* is present in our environment, patients could inhale resistant spores and subsequently develop azole-resistant disease. Indeed, azole-resistant aspergillosis was reported in azole-naïve patients, indicating that resistance does not exclusively develop during azole therapy (24).

Favorable conditions for resistance development are exposure to azole compounds and the presence of reproducing fungus (1). *A. fumigatus* is abundantly present in our environment as saprophytic, reproducing fungi, most notably in soil and compost. Furthermore, azoles are commonly used for plant protection as well as material preservation. Therefore, it appears that resistance development in *A. fumigatus* is feasible in the environment, and isolates that develop resistance to fungicides might be cross-resistant to medical triazoles.

We investigated if *A. fumigatus* isolates that are present in

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TABLE 1. Characteristics of 15 ITZ$^+$ and 15 ITZ$^-$ environmental *A. fumigatus* isolates

Category	Isolate no.	Source of isolate	Year of isolation	MIC (mg/liter)[a]						Cyp51A substitution[b]		
				ITZ	VCZ	POS	AMB	TER	CAS	TR	Codon 98	Other(s)
ITZ$^+$	V17-24	Air sample, patient room	2002	>16	1	0.5	0.5	0.031	0.25	+	L98H	S297T, F495I
	V22-76	Air sample, patient room	2003	16	16	0.5	0.5	0.031	0.25	+	L98H	
	V37-56	Air sample, patient room	2005	>16	2	0.5	1	0.125	0.25	+	L98H	
	V61-56	Water filter sample	2007	>16	4	0.5	0.5	0.25	1	+	L98H	
	V61-57	Water filter sample	2007	>16	4	0.5	0.5	0.063	0.5	+	L98H	
	V61-50	Soil	2007	>16	4	0.5	0.5	0.25	>32	+	L98H	
	V61-79	Soil	2007	>16	8	0.5	1	0.016	0.5	+	L98H	
	V62-05	Soil	2007	>16	8	0.5	0.5	0.125	0.25	+	L98H	
	V62-09	Soil	2007	>16	4	0.5	1	0.063	1	+	L98H	Q141H
	V62-10	Soil	2007	>16	4	0.25	1	0.125	0.5	+	L98H	
	V62-14	Soil	2007	>16	4	0.25	1	0.063	1	−		
	V62-48	Seeds	2007	>16	4	0.5	0.5	0.25	1	+	L98H	S52T
	V62-63	Compost	2007	>16	>16	0.5	1	0.0031	0.5	+	L98H	
	V62-79	Soil	2007	>16	0.5	0.25	0.5	0.5	0.5	−		
ITZ$^-$	V04-22	Air sample, patient room	2000	0.25	0.5	0.063	1	0.063	0.5	−		
	V22-74	Air sample, patient room	2003	0.25	0.5	0.031	1	0.063	0.5	−		
	V22-80	Air sample, patient room	2003	0.25	0.5	0.031	1	0.5	0.5	−		
	V57-35	Water filter sample	2007	0.25	0.5	0.031	1	0.063	0.5	−		
	V57-53	Water filter sample	2007	0.25	0.5	0.063	0.5	0.016	0.5	−		
	V62-04	Soil	2007	0.25	1	0.125	0.5	0.25	0.5	−		
	V62-06	Soil	2007	1	4	0.25	1	0.5	0.5	−		F46Y, M172V, E427K
	V62-07	Soil	2007	0.5	0.5	0.125	1	0.063	1	−		
	V62-12	Soil	2007	0.5	4	0.25	1	0.031	1	−		F46Y, M172V, E427K
	V62-13	Soil	2007	1	2	0.25	1	0.5	1	−		
	V62-23	Soil	2007	0.25	1	0.063	1	0.25	0.5	−		
	V63-01	Soil	2007	0.25	0.5	0.031	0.5	0.125	0.5	−		
	V63-03	Soil	2007	0.25	1	0.063	1	0.063	0.5	−		
	V63-04	Soil	2007	0.25	0.5	0.063	0.5	0.016	0.5	−		
	V63-05	Soil	2007	0.5	4	0.25	0.5	0.5	0.5	−		

[a] VCZ, voriconazole; POS, posaconazole; AMB, amphotericin B; TER, terbinafine; CAS, caspofungin.

our environment are resistant to medical triazoles and if they are cross-resistant to azole fungicides. Furthermore, we characterized the isolates by microsatellite typing in order to determine if they were genetically related to clinical *A. fumigatus* isolates previously obtained from patients cared for in our University Medical Center.

MATERIALS AND METHODS

We searched our private fungus culture collection for *A. fumigatus* isolates that had been cultured from the hospital indoor environment. Most indoor environment isolates were obtained from air sampling of patient rooms. These isolates had been stored in 10% glycerol at −80°C. In addition, samples were collected from soil sampled from the direct surroundings of the University Medical Center, from soil obtained from plant nurseries and from leaves, seeds, and compost obtained from a garden center. Environmental sampling was carried out in the months of July and August of 2007.

To recover *A. fumigatus* isolates from the outdoor environment, 2 g of soil, pulverized leaves, or pulverized seeds was dissolved in 8 ml 0.2 M NaCl–1% Tween 20. From this suspension, 100 μl was plated on Sabouraud dextrose agar, and plates were supplemented with 4 mg/liter of ITZ and incubated at 37°C. For each *A. fumigatus* isolate that grew on the ITZ-containing agar (an ITZ$^+$ isolate), an azole-susceptible *A. fumigatus* isolate (ITZ$^-$) that had been cultured from the same environment (outdoor or indoor) was selected.

Isolates were tested for their antifungal susceptibility to ITZ, voriconazole, posaconazole, amphotericin B, terbinafine, and caspofungin using the CLSI M38-A broth microdilution reference method (15). In addition, the activity of five azole fungicides, including cyproconazole, metconazole, thiabendazole, tebuconazole, and penconazole, was determined using the same method.

Molecular methods were used for strain identification, determination of the resistance mechanism, and for genotyping. For this, conidia from each strain were inoculated into 15 ml of GYEP medium (2% glucose, 0.3% yeast extract, 1% peptone) and grown for 48 h at 37°C. Mycelial mats were recovered, dried, and subjected to a DNA isolation protocol (20). In order to rule out any cryptic species within *Aspergillus* section *Fumigati*, molecular identification was performed by amplification of parts of the β-tubulin gene and calmodulin gene (10, 12). DNA sequences were determined using a BigDye Terminator version 3.1 cycle sequencing kit (ABI) and an ABI 3100 DNA sequencer. Sequence alignments were performed using CLUSTAL-X, and the neighbor-joining method was used for the phylogenetic analysis (16, 19). Sequences were compared with those of *Aspergillus fumigatus*, *Aspergillus lentulus*, *Aspergillus viridinutans*, *Aspergillus brevipes*, *Aspergillus novofumigatus*, *Aspergillus fumigatiaffinis,* and *Neosartorya* species, all obtained from the CBS Fungal Biodiversity Centre, Utrecht, The Netherlands. The full coding sequence of the *cyp51A* gene was determined by PCR amplification and subsequent sequencing (9). For analysis, the *cyp51A* sequence under accession number AF338659 in GenBank was used for comparison to detect mutations.

Microsatellite genotyping was used to determine the genetic relatedness between the environmental isolates described for *A. fumigatus* with a short tandem repeat *A. fumigatus* assay (8). From six loci, consisting of three tri- and three tetranucleotide repeats, fragments were amplified by using fluorescently labeled primers (8). The sizes of the fragments were determined by addition of the GeneScan LIZ[500] marker and subsequent analysis of the fragments on the Applied Biosystems 3730 DNA analyzer. Assignment of repeat numbers in each marker was determined from the GeneScan data by using the Peak Scanner version 1.0 software (Applied Biosystems). The repeat numbers of the environmental isolates were compared to those of our previously analyzed collection of 32 ITZ$^+$ and 32 ITZ$^-$ clinical *A. fumigatus* isolates cultured between 2000 and 2007 (19). Allele-sharing distance matrices were generated from the tandem repeat numbers and were used as input to the Neighbor program of the PHYLIP version 3.6 software package to produce dendrograms (2, 19).

To test for significant differences between antifungal susceptibilities, the *t* test was used on the natural logarithm of the MICs.

RESULTS

A total of 248 *A. fumigatus* isolates, cultured from the hospital indoor environment, were present in our fungus culture collection. These isolates had been cultured between 1995 and 2007, and five isolates grew on the ITZ-containing agar slants (ITZ$^+$): three isolates from air sampling of patient rooms in the hematology ward in 2002, 2003 and 2005 and two from hospital water in 2007 (Table 1).

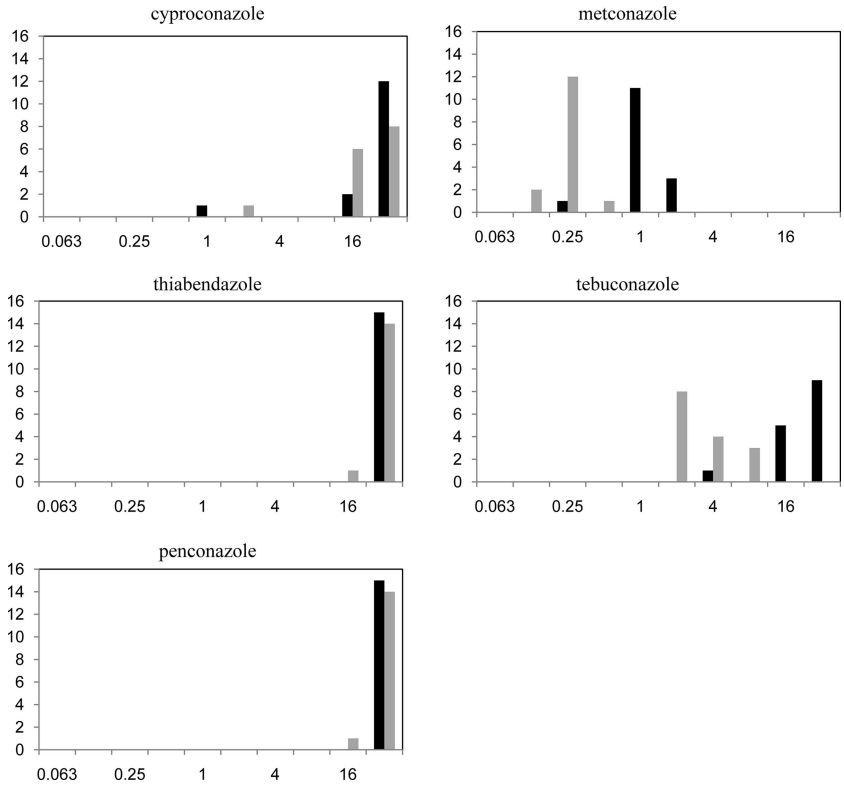

FIG. 1. In vitro activity of five azole fungicides against 15 ITZ-resistant (black bars) and 15 ITZ-susceptible (gray bars) *A. fumigatus* isolates. The number of isolates is given on the *y* axis, and the MIC (mg/liter) on the *x* axis. Those isolates that were not inhibited by a concentration of 16 mg/liter were assigned to the >16 mg/liter category.

A. fumigatus was also cultured from 49 of 79 outdoor environmental samples. Culture of soil samples taken from the direct proximity of the hospital showed the presence of ITZ$^+$ *A. fumigatus* isolates in six samples obtained from flower beds. Cultures from natural soil were *A. fumigatus* positive, but these were never ITZ resistant. Following this observation, we cultured compost used in the flower beds as well as seeds, leaves, and compost that we obtained from a garden center and from a plant nursery, both located in towns other than the hospital. ITZ$^+$ isolates were recovered from seeds (one isolate), compost from the plant nursery (one isolate), and commercial compost from the garden center (two isolates). Control isolates (ITZ$^-$) were matched 1:1 to the environment from which an ITZ$^+$ isolate was cultured. All 15 ITZ$^+$ isolates were confirmed to be highly resistant to ITZ (MIC of >16 mg/liter) and showed reduced susceptibility to voriconazole (median MIC of 4 mg/liter; range of 0.5 to >16 mg/liter) and posaconazole (median MIC of 0.5 mg/liter; range of 0.25 to 1 mg/liter) compared to the ITZ$^-$ control isolates ($P < 0.005$). The median MICs for ITZ$^-$ isolates were 0.25 mg/liter for ITZ, 0.5 mg/liter for voriconazole, and 0.063 for posaconazole (Table 1). No significant differences in MICs were observed for amphotericin B ($P = 0.285$), terbinafine ($P = 0.909$), and caspofungin ($P = 0.610$). Three fungicides, cyproconazole, thiabendazole, and penconazole, showed no in vitro activity against the ITZ$^-$ and ITZ$^+$ *A. fumigatus* isolates (Fig. 1). Metconazole and tebuconazole showed activity against ITZ$^-$ isolates, with median MICs of 0.25 mg/liter and 2 mg/liter, respectively. These compounds were significantly less active against ITZ$^+$ isolates, with the median MIC shifting to 1 mg/liter for metconazole and to >16 mg/liter for tebuconazole ($P < 0.005$) (Fig. 1).

Sequencing of the *cyp51A* gene showed the presence of two alterations, a 34-bp tandem repeat in the promoter region combined with the presence of a mutation that led to a substitution at codon 98 of leucine to histidine (TR/L98H), in 13 of 15 (86%) ITZ$^+$ isolates, which was identical to the mechanism previously observed with the majority of resistant clinical isolates (19). No changes were found in the *cyp51A* gene of the two other ITZ$^+$ isolates, indicating another yet unknown mechanism of resistance, and the TR/L98H alterations were absent in all of the 15 matched ITZ$^-$ control isolates.

All ITZ$^+$ and ITZ$^-$ isolates were identified as *A. fumigatus* by sequence analysis of the β-tubulin and calmodulin genes. Microsatellite typing of six STR loci showed that all isolates were genetically unique. Comparing the genetic relatedness by generating dendrograms of the STR profiles showed that the ITZ$^+$ environmental isolates clustered apart from ITZ$^-$ environmental isolates. Furthermore, the ITZ$^+$ environmental isolates were found to cluster with the previously analyzed azole-resistant clinical isolates. Likewise the ITZ$^-$ environmental isolates clustered together with the azole-susceptible clinical isolates in the remaining clusters. Two out of the three azole-resistant isolates without the dominant TR and L98H mutations clustered in azole-susceptible clusters (Fig. 2) (19).

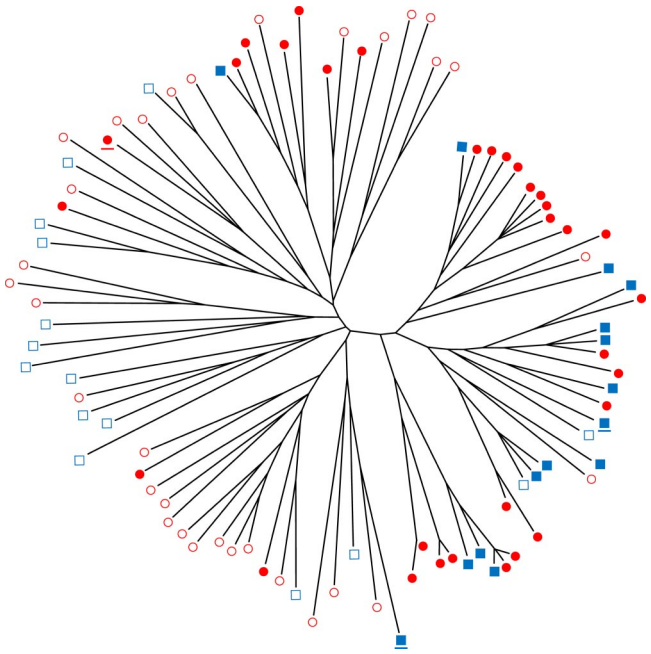

FIG. 2. Genotypic relatedness of clinical and environmental azole-resistant *A. fumigatus* isolates. The figure shows genetic distances of 15 environmental ITZ-resistant *A. fumigatus* isolates (blue squares, filled), 15 environmental control *A. fumigatus* isolates (blue squares, open), 32 clinical ITZ-resistant *A. fumigatus* isolates (red circles, filled), and 32 clinical control *A. fumigatus* isolates (red circles, open). All resistant isolates were found to have the L98H substitution and a tandem repeat in the promoter region of the *cyp51A* gene, except for the underlined isolates.

DISCUSSION

Our study provides for the first time evidence that patients with invasive aspergillosis due to azole-resistant *A. fumigatus* might acquire the fungus from the environment. We were able to culture *A. fumigatus* isolates, resistant to ITZ, from the indoor environment as well as the outdoor environment, with soil and air samples from patient rooms being positive. The emergence of resistant isolates in the hospital environment coincided with that observed with clinical isolates, i.e., after the year 1999 (19). The majority of indoor and outdoor environmental azole-resistant isolates exhibited a phenotype and mechanism of resistance that were identical to the main type found for resistant isolates recovered from clinical samples of patients admitted to our hospital. Also, microsatellite typing of the isolates showed that the environmental and clinical azole-resistant isolates clustered together, indicating genetic relatedness and possibly a common ancestor.

We used ITZ-containing Sabouraud agar to select for resistant isolates, which possesses the theoretical risk of induction of resistance in the *A. fumigatus* isolates due to exposure to ITZ. However, we regard this as highly unlikely, as the incubation (exposure) period was short, i.e., a maximum of 48 h, and most resistant isolates contained two genomic changes, both of which are required for the resistant phenotype (14). We have never found either of these changes separately in azole-susceptible isolates. In addition, we have performed induction experiments with wild-type isolates by repeated exposure to ITZ but thus far have been unsuccessful in inducing laboratory mutants containing the TR/L98H resistance mechanism (unpublished observations), which is similar to the experience reported by others (7). Detection of resistance mechanisms directly with the original sample, i.e., soil or compost, for instance, by a specifically designed PCR, would overcome a selection step that involves exposure of the isolates to azole compounds.

The dominance of a single resistance mechanism and the genetic relatedness between clinical and environmental isolates indicate that acquisition of azole-resistant isolates from the environment is the most important infection route, more important than the generally recognized mode of resistance development during azole therapy of patients with aspergillus disease. The presence of *A. fumigatus* resistant to medical triazoles in our environment yields a major threat for immunocompromised patients, as it is unclear if (further) spread of resistant isolates can be prevented and alternative treatment options are limited, especially for invasive disease that has spread to the central nervous system (21).

An important question is how resistance to medical triazoles arises in environmental isolates. Although our study does not address this issue, we have previously suggested the use of azole fungicides for plant protection and material preservation as a possible cause (19, 24). Azoles are widely used preharvest in grain-growing and grass-growing environments as well as postharvest to prevent spoilage (11). Also, azoles are used for preservation of materials, such as paints, coatings, and wall paper pastes, and are routinely applied to mattresses in order to prevent fungal growth. In the present study, the azole-resistant *A. fumigatus* isolates that we cultured from the outdoor environment were found exclusively in soil from flowerbeds. In natural soil, *A. fumigatus* was recovered, but azole resistance was never observed. Since the flowerbeds contained both compost and cultivated plants, we went on to culture seeds, leaves, and compost which we obtained from a plant nursery and a garden center and which were also found to contain azole-resistant isolates. Fungicides are used during the production of plants, and residues might be present in organic matter that is used for composting. Azole fungicides are recognized to have the potential to persist in soil (4, 5, 18), and fungicide residues have been detected in compost (23). Furthermore, resistance to azole fungicides has been reported for numerous plant pathogenic molds, such as *Mycosphaerella graminicola* (6). As *A. fumigatus* is a thermotolerant fungus and readily grows in compost heaps, resistance to azole fungicides might arise under these conditions. Stepwise increased exposure of the maize pathogen *Colletotrichum graminicola*, a fungus also capable of causing cutaneous mycosis and keratitis in humans, to the fungicide tebuconazole showed loss of activity by this triazole as well as by ITZ and voriconazole, indicating that fungicides are capable of inducing resistance to medical azoles (17).

If fungicides are responsible for the emergence of resistance to medical triazoles in *A. fumigatus*, a resistance mechanism would have developed that causes primarily resistance to the fungicide. Resistance to other azole compounds, such as medical triazoles, can be expected if these compounds share the same target or have a similar molecule structure. The suspected fungicide or group of fungicides will be active against ITZ^- isolates but will exhibit reduced activity against ITZ^+

isolates. Among the five fungicides that we have investigated in this study, three exhibited no activity, while two were active against the ITZ^- isolates. Both of these, metconazole and tebuconazole, were significantly less active against the ITZ^+ isolates, indicating that the TR/L98H substitution results in a cross-resistant phenotype, which supports a possible role for fungicides in resistance development. As azole fungicides have been used for many decades, further studies are required to understand their role in the emergence of resistance. It is important to investigate the temporal relationship between the year of introduction of fungicides and the presence of cross-resistance with medical triazoles. This would require the testing of all azole fungicides licensed for use in The Netherlands, prior to the emergence of azole resistance in *A. fumigatus* in the year 2000. Also, laboratory experiments that determine the ability of azole fungicides to induce mutations in the *cyp51A* gene, most notably the TR/L98H substitution, would prove such a relationship.

We therefore believe that our findings could be explained by a relationship between environmental azole use and development of cross-resistance to medical triazoles. The intensive use of triazole fungicides creates an environment that allows the TR/L98H strains to survive in our environment. Spread of the TR/L98H and possibly other azole resistance mechanisms cannot be excluded. In addition to the abovementioned research and international surveillance studies of clinical isolates, environmental sampling is required in order to determine and track the spread of azole resistance in the environment.

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